Journal of Extracellular Vesicles

ISSN: (Print) 2001-3078 (Online) Journal homepage: https://www.tandfonline.com/loi/zjev20

Highlights of the São Paulo ISEV workshop on extracellular vesicles in cross-kingdom communication

Rodrigo P. Soares, Patrícia Xander, Adriana Oliveira Costa, Antonio Marcilla, Armando Menezes-Neto, Hernando Del Portillo, Kenneth Witwer, Marca Wauben, Esther Nolte-`T Hoen, Martin Olivier, Miriã Ferreira Criado, Luis Lamberti P. da Silva, Munira Muhammad Abdel Baqui, Sergio Schenkman, Walter Colli, Maria Julia Manso Alves, Karen Spadari Ferreira, Rosana Puccia, Peter Nejsum, Kristian Riesbeck, Allan Stensballe, Eline Palm Hansen, Lorena Martin Jaular, Reidun Øvstebø, Laura de la Canal, Paolo Bergese, Vera Pereira-Chioccola, Michael W. Pfaffl, Joëlle Fritz, Yong Song Gho & Ana Claudia Torrecilhas

To cite this article: Rodrigo P. Soares, Patrícia Xander, Adriana Oliveira Costa, Antonio Marcilla, Armando Menezes-Neto, Hernando Del Portillo, Kenneth Witwer, Marca Wauben, Esther Nolte-`T Hoen, Martin Olivier, Miriã Ferreira Criado, Luis Lamberti P. da Silva, Munira Muhammad Abdel Baqui, Sergio Schenkman, Walter Colli, Maria Julia Manso Alves, Karen Spadari Ferreira, Rosana Puccia, Peter Nejsum, Kristian Riesbeck, Allan Stensballe, Eline Palm Hansen, Lorena Martin Jaular, Reidun Øvstebø, Laura de la Canal, Paolo Bergese, Vera Pereira-Chioccola, Michael W. Pfaffl, Joëlle Fritz, Yong Song Gho & Ana Claudia Torrecilhas (2017) Highlights of the São Paulo ISEV workshop on extracellular vesicles in cross-kingdom communication, Journal of Extracellular Vesicles, 6:1, 1407213, DOI: 10.1080/20013078.2017.1407213

To link to this article: https://doi.org/10.1080/20013078.2017.1407213

Published online: 26 Nov 2017.

JOURNAL OF EXTRACELLULAR VESICLES, 2017
VOL. 6, 1407213
https://doi.org/10.1080/20013078.2017.1407213

Taylor & Francis
Taylor & Francis Group

MEETING REPORT

OPEN ACCESS Check for updates

Highlights of the São Paulo ISEV workshop on extracellular vesicles in cross-kingdom communication

Rodrigo P. Soares[a], Patrícia Xander[b]*, Adriana Oliveira Costa[c], Antonio Marcilla[d,e], Armando Menezes-Neto[a], Hernando Del Portillo[f,g], Kenneth Witwer[h], Marca Wauben[i], Esther Nolte-`T Hoen[i], Martin Olivier[j], Miriã Ferreira Criado[k], Luis Lamberti P. da Silva[k], Munira Muhammad Abdel Baqui[k], Sergio Schenkman[l], Walter Colli[m], Maria Julia Manso Alves[m], Karen Spadari Ferreira[b], Rosana Puccia[l], Peter Nejsum[n], Kristian Riesbeck[o], Allan Stensballe[p], Eline Palm Hansen[q], Lorena Martin Jaular[r], Reidun Øvstebø[s], Laura de la Canal[t], Paolo Bergese[u], Vera Pereira-Chioccola[v], Michael W. Pfaffl[w], Joëlle Fritz[x], Yong Song Gho[y] and Ana Claudia Torrecilhas[b]

[a]Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte; [b]Laboratório de Imunologia Celular e Bioquímica de Fungos e Protozoários, Departamento de Ciências Farmacêuticas, UNIFESP, São Paulo, Brazil; [c]Departamento de Analises Clinicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; [d]Área de Parasitología, Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, Universitat de València, Valencia, Spain; [e]Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, Health Research Institute-La Fe, Universitat de València, Valencia, Spain; [f]ICREA, Barcelona, Spain & ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), Hospital Clínic - Universitat de Barcelona, Barcelona, Spain; [g]Institut d'Investigació Germans Trias i Pujol (IGTP), Badalona, Spain; [h]Department of Molecular and Comparative Pathobiology, and Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA; [i]Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [j]Department of Microbiology and Immunology, McGill University, Montréal, Canada; [k]Department of Cell and Molecular Biology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil; [l]Departamento de Microbiologia, Imunologia e Parasitologia, UNIFESP, São Paulo, Brazil; [m]Departamento de Bioquímica, IQ, USP, São Paulo, Brazil; [n]Department of Clinical Medicine, Faculty of Health, Aarhus University, Aarhus, Denmark; [o]Clinical Microbiology, Department of Translational Medicine, Lund University, Malmö, Sweden; [p]Aalborg University, Aalborg, Denmark; [q]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [r]Institut Curie, PSL Research University INSERM U932, Paris, France; [s]Head of the Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway; [t]University of Mar del Plata, Mar del Plata, Argentina; [u]Department of Molecular and Translational Medicine, INSTM, CSGI University of Brescia, Brescia, Italy; [v]Adolfo Lutz Institute, São Paulo, Brazil; [w]Department of Animal Physiology & Immunology, School of Life Science, Technical University of Munich, Freising Weihenstephan, Germany; [x]Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Campus Belval, Luxembourg; [y]Department of Life Sciences, POSTECH (Pohang University of Science and Technology), Pohang, South Korea

ABSTRACT

In the past years, extracellular vesicles (EVs) have become an important field of research since EVs have been found to play a central role in biological processes. In pathogens, EVs are involved in several events during the host–pathogen interaction, including invasion, immunomodulation, and pathology as well as parasite–parasite communication. In this report, we summarised the role of EVs in infections caused by viruses, bacteria, fungi, protozoa, and helminths based on the talks and discussions carried out during the International Society for Extracellular Vesicles (ISEV) workshop held in São Paulo (November, 2016), Brazil, entitled Cross-organism Communication by Extracellular Vesicles: Hosts, Microbes and Parasites.

ARTICLE HISTORY
Received 3 November 2017
Accepted 11 November 2017

KEYWORDS
Extracellular vesicles; infectious diseases; pathogens; cell communication; isolation

The ISEV satellite meeting

Extracellular vesicles (EVs) are prokaryotic and eukaryotic cellular components involved in communication and in several processes ranging from infectious to inflammatory diseases [1,2]. The structural and functional characterisation of EVs has been focused on vesicles isolated from the extracellular milieu of cultured cells in normal or pathological conditions and from microorganisms. The general term extracellular vesicle has currently been proposed to include exosomes, microvesicles, and apoptotic bodies, which vary in size, number, and biogenesis [3].

Following a growing interest in the EV field for 10 years, a group of scientists created the International Society for Extracellular Vesicles (ISEV), following the International Workshop on Exosomes organised in Paris in January 2011. ISEV is a global society, whose mission relies on spreading education,

CONTACT Ana Claudia Torrecilhas ana.trocoli@gmail.com Universidade Federal de São Paulo, Laboratório de Imunologia Celular e Bioquímica de Fungos e Protozoários, Rua São Nicolau, 210, Diadema, SP 09913-030, Brazil
*These authors contributed equally to this work.

communication, and science among the different levels of academia. Since then, six annual meetings promoted by the Society were held in different cities including Göteborg (2012), Boston (2013), Rotterdam (2014, 2016), Washington D.C. (2015), and Toronto (2017). ISEV has also promoted satellite workshops that congregated 40–60 participants and have so far occurred in New York (2012), Melbourne (2014), Budapest (2013), and Singapore (2015), among others. Considering that infectious diseases are a major health problem in Brazil and other tropical countries, an ISEV workshop focused on EVs from pathogens was held in São Paulo, Brazil, in 2016. This meeting was organised by local groups working in infectious disease-related EVs and ISEV Board and had the participation of scientists and students coming from many Brazilian cities as well as other countries. The main topics included: EVs isolation and characterisation; EVs in viruses, bacteria, fungi, protozoa and helminths; the role of EVs in pathogen–host interactions. Before the meeting, an Education Day including introductory talks to graduate students and postdocs was organised. Below we summarised the highlights of the event with a brief background on the EVs role in host–pathogen interactions.

The role of pathogen EVs in the interaction with the host

During evolution, human pathogens have evolved complex life cycles that often involve different hosts at intracellular or extracellular residencies, and that evoked a wide range of clinical outcomes from asymptomatic infections to severe disease, including death [4]. Host–pathogen interactions thus require fine-tuning intercellular communication adapted to the particularities of each microorganism for which EVs seem to play a key role [1,5]. In fact, since the molecular composition of EVs seems to comprise both random and selected cargo [6], EVs are able to transfer functional information to recipient cells that signal normal and pathophysiological processes. Moreover, as such selected cargo contains pathogen-derived molecules, EVs also represent novel approaches for identifying novel biomarkers of disease, and for discovering antigens for vaccination [7].

Several independent observations have demonstrated that pathogen-derived EV cargos seem to be highly secreted in small EVs coming from virus infected cells [7]. Yet, we are far from understanding the mechanism(s) involved in this selective sorting. On the other hand, this is not so obvious for pathogen EVs in infections caused by bacteria, fungi or parasites. In *Mycobacterium tuberculosis*, the machinery for ubiquitination was implicated in incorporation of bacterial proteins into exosomes [8], although it is unknown whether this mechanism is also used by other bacteria or microorganisms. Clearly, studies on the mechanisms for sorting selective pathogen-derived cargo into EVs might pave the way for discovering inhibitors of such mechanisms.

Another major topic in host–pathogen interaction is functional studies on the effect of transferring information to recipient cells. Several examples currently support that this transfer might facilitate the colonisation of niches for pathogen invasion, exacerbate clinical symptoms, and trigger immune responses, among other effects [9,10]. A recent report on *Paracoccidioides brasiliensis*, which causes major fungal lung complications, shows the importance of EVs in the intercellular communication with the host cells [11]: there was a dose-dependent effect on the production of murine macrophage pro-inflammatory mediators upon incubation with fungal EVs, which induced a potent and specific M1 polarisation. Moreover, EVs stimulated conversion of non-protective M2-macrophages to protective M1-polarised cells with higher fungicidal activity. Several other studies show the pathogen EV elicitation of immune responses in human parasitism that directly affect the outcome of infection [12]. Unveiling mechanistic insights of such outcomes will provide the basis for developing alternative control strategies.

A great deal of research on pathogen EVs is directed to their use as novel therapeutic agents [13]. Indeed, since the original work on the demonstration that dendritic cell-derived exosomes primed with *Mycobacterium* antigens were able to confer protection, there are growing examples of the EV use as vaccines in other bacterial, fungal, and parasitic infections [12]. The major challenge ahead, however, is to understand the mode-of-action of EV-based vaccines. A significant advance on this relevant topic has recently been published using a mouse malaria model [14], where immunisation with exosomes obtained from infected reticulocytes evoked long-lasting protection from a lethal challenge with *Plasmodium falciparum*. In that model, splenectomy before immunisation abolished the protective effect of vaccination, but immunisation elicited effector memory T-cells that, unlike effector memory T-cells from experimental infection, were not exhausted. Further understanding of the mechanism of action of EV-based vaccines will guide more rational efforts in developing cell-free vaccines against pathogens, and will speed-up regulatory issues for their use in human clinical trials.

In terms of diagnostics, even though the EV molecular cargos of different pathogens are well known [1],

there are few examples of their use as infection biomarkers. Yet, a recent report on the characterisation of miRNAs from *Schistosoma mansoni* EVs in human samples demonstrated their potential in this growing research area [15]. In summary, research on the biogenesis, molecular composition, and function of EVs in host-pathogen interactions is a growing field with clear translational implications for eradicating infectious diseases that affect the human kind.

Protocols for pathogens EV purification and characterisation

Working with pathogen EVs can be very challenging since each microorganism has peculiarities that are inherent to its biology. Pathogenic microorganisms may develop inside host cells, in the extracellular matrix, circulate in the body fluids, depending on the species, developmental stage, and mode of transmission. Some pathogens are transmitted by arthropod vectors such as protozoan parasites, and vary according to the host environment, or culture conditions. Common changes are alterations in the composition and cell walls and the type of secreted materials. All of these will affect EV release. Moreover, the release of EVs can be affected by multiple host factors, as is the case of helminths. In this context, the protocols for EVs isolation from pathogens should be standardised according to the cell model and to the peculiarities of the organism. Another major concern is that each organism releases different types of vesicles with unique characteristics including size profile. Therefore, how to separate and characterise EVs and define "gold standards" from pathogens is a major challenge.

Over the past years a range of methods to separate and probe EVs have been assessed, making EV preparations available in increasing amounts. Paolo Bergese (Universitá degli Studi di Brescia, Italy) showed a COlloidal NANoplasmonic (CONAN) assay for purity assessment and titration of EVs [16], which used in combination with atomic force microscopy (AFM) and helium ion microscopy (HIM) allowed to assess the impact of the purity of EV preparations on their biological activity [16]. Bergese also points out that colloidal properties of EVs (such as size, stiffness, surface charge, interfacial energy, hydrodynamic behaviour, adhesion, spreading) might play a major role in the case of communication networks belonging themselves to the biocolloidal domain, such as microbiota populations Joel Rozowsky (Yale University, USA) showed the exceRpt pipeline (github.gersteinlab.org/exceRpt) of the Extracellular RNA Communication Consortium (ERCC) has for several years allowed users to analyse small-RNA-seq data from extracellular preparations. A major feature of the pipeline is a comprehensive series of quality filters. Lorena Martin-Jaular (Institute Curie, France) spoke on the "diversity of vesicles and rigor in isolation." Beginning with the Théry laboratory's discoveries on the protein components of EVs with different sizes and densities [17], Lorena Martin-Jaular then shared her experience with purification of EVs from HIV-1 infected Jurkat T-cells using Iodixanol velocity gradients. EVs are present in the lighter fractions, while HIV virions predominant in the heavier fractions. She observed slightly less syntenin, possibly also CD9, in virus fractions. Acetylcholinesterase, often used as a surrogate marker of EVs, was found not to be a reliable marker: it was present only in the lightest fractions and had only minimal overlap with EVs.

Laura de la Canal (University of Mar del Plata) gave a second overview talk, "Lessons from plants and fungi." De la Canal related her experience with the challenges of investigating EVs in organisms in which EVs have not yet been well studied [18,19]. Investigators in uncharted waters can expect resistance and only slow recognition of new findings. Vilma Regina Martins (A.C.Camargo Cancer Center, Brazil) discussed regulation of exosome secretion. André Vettore (UNIFESP, Brazil) used a Cholera Toxin B and Annexin V to affinity purifies EVs from cancer patient samples, finding various markers for cisplatin resistance. Next presentation, Joel Rozowsky (Yale University, USA) introduced the audience to the US NIH Extracellular RNA Communication Consortium's exceRpt pipeline for exogenous exRNA analysis. Allan Stensballe (Aalborg University, Denmark) shared his experience with proteome analysis of non-eukaryotic EVs.

Virus

Viruses are small infectious particles that require a host cell to survive and multiply. A distinguished feature of viruses is that these agents directly affect many intracellular pathways of their host cells. It is now clear that the mechanisms involved in EV biogenesis described for mammalian cells share many similarities with that of assembly and budding of many enveloped viruses [6,20]. In addition, several types of non-enveloped viruses have been shown to exit intact cells via enclosure in EVs, which allows hiding from the immune system [21]. Virus infection can influence the EV population released from infected cells by manipulating EV production with the incorporation of proteins and nucleic acids from both host and viral origin into those EVs. Therefore, enclosure of virions and viral components in EVs can impact host-virus interactions and antiviral immunity. EVs from virally infected cells may

also help to enhance viral propagation both via receptor-dependent and receptor-independent mechanisms and promote the possibility of genetic exchange between the viral species, which will enhance the fitness of the overall population.

Despite the progress in the field, the mechanisms by which viruses affect the molecular composition and release of EVs still need to be clarified, and the knowledge obtained can help the development of better antiviral therapies and vaccines. Several studies were presented in the ISEV workshop focusing on the biogenesis, release and purification of EVs in the context of infection by different viruses, such as HIV, Dengue, HTLV-1, and EMCV. In most studies, differential centrifugation of culture supernatant is applied, followed by further purification on iodixanol density or velocity gradients and probing with specific antibodies against virus molecules and EV markers. The presented data indicated that it is particularly difficult to discriminate enveloped viruses from EV due to the lack of discriminating protein markers and overlap in their buoyant density. Most likely, the population of membrane-enclosed particles released by the infected cells is highly heterogenous, containing EV with graded levels of host and viral factors and replication-capable viruses.

Naked viruses, on the contrary, can be separated from EV and EV-enclosed viruses based on buoyant density. However, more advanced methods will need to be developed to be able to analyze the heterogenous mix EV released by infected cells, which may consist of constitutively released EV, EV that contain infectious virus, and infection-induced EV that do not contain virus. Being able to purify these different EV types based on discriminatory markers will help to assess the contribution of each of these EV types to virus progression or antiviral immunity.

Kenneth Witwer (Johns Hopkins University, USA) began the session with an overview of virus and EV diversity and how many of the challenges faced in the virology field also apply to EV studies. EVs and enveloped viruses like HIV share biogenesis pathways [22,23] and are difficult to separate [24,25], similar to the case for EVs and lipoproteins [26]. Findings on virus entry into cells may provide clues as to how EVs deposit cargo into cells [27]. A cautionary note was sounded for combined studies of EVs and viruses, as EV-depleted culture conditions were found to influence production of retroviruses, as well [28]. Other presentations in this session included the work of Sharon Martins (FIOCRUZ, Brazil), on dendritic cells (DCs) and dengue virus. In dengue, an initial infection can be followed by re-infection by different serotypes, eliciting a cytokine storm and antibody-dependent virus replication. In her studies, Martins examined two dengue strains with different outcomes: 290 and 5532 (DENV3). These result in fever without complication and fatal bleeding, respectively. Martins reported that DC EVs may protect a subpopulation of viruses by engulfing or aggregating them. Furthermore, miRNAs associated with immune evasion were differentially expressed during exposure to the different viruses.

Bacteria

EVs from Gram-negative bacteria are known as outer-membrane vesicles (OMVs) but this denomination is not commonly applied to Gram-positive bacteria or *Mycobacteria* [29]. OMVs are crucial for intercellular, interspecies, and interkingdom communication; however, their biogenesis is not completely understood. OMV contents and surface components are highly variable, often involved in virulence and may include proteins, phospholipids such as lipopolysaccharide (LPS), peptidoglycan, and nucleic acids depending on the species/strains [30]. The LPS is a strong TLR4 agonist with importance in the pro-inflammatory events following bacterial infection [31–34]. OMV DNA contents activate TLR9 that also trigger inflammatory responses [2,35].

Joëlle Fritz (University of Luxembourg, Luxembourg) presented results showing that OMVs released from *Salmonella enterica*, contains small RNA that may be able to modulate the human immune system. Tonya Duarte (IFBA, Brazil) characterised morphologically EVs released from macrophages infected with *M. tuberculosis* and showed that these EVs also modulate host immune response. Reiudun Øvstebøo (Oslo University Hospital, Norway) showed that microparticle-associated tissue factor activity correlates with plasma levels of bacterial lipopolysaccharides in patients with meningococcal septic shocks of meningococcal meningitis present in the plasma of patients contributed to the disseminated vascular pathogenesis. Kristian Riesbeck (Lund University, Sweden) presented results indicating that OMVs from *Moraxella catarrhalis* and *Haemophilus influenzae* affected the immune system and can carry β-lactamases that induce increased survival of other bacteria. Infection of salmons was also correlated in the work of Hanne C. Winther-Larsen (University of Oslo, Norway) with the type and the capacity of OMVs released by *Piscirickettsia salmonis* to upregulate several proinflammatory genes.

The protocols developed to isolate bacterial EVs usually involve differential centrifugation, filtration, and ultracentrifugation [35,36]. A summary of the multiple roles, isolation, characterisation and use of pathogenic bacteria was illustrated by Yong Song Gho (POSTECH,

Republic of Korea) available in a web portal (http://student4.postech.ac.kr/bevpedia/xe/xe/.) However, proteomic studies revealed that ultracentrifugation does not completely remove bacterial contaminant, such as protein aggregates or membrane debris, and some authors have proposed to use density gradient ultracentrifugation to obtain cleaner EV preparations [37]. Transmission electron microscopy (TEM) is generally used to confirm EV purification since there are no specific antibodies or probes for labelling bacterial EVs. This and other aspects concerning the study of bacterial OMVs/EVs have been highly covered in the meeting because they need to be overcome to ensure reliability of the results. For example, Michael W. Pfaffl (Technical University of Munich, Germany) discussed multiple isolation and characterisation methods from different bacterial and food sources in farm animals. The research aims to get more insights in the communication via OMVs/EVs of a healthy and diseased microbiome in food-producing animals. Goal is to increase animal health and welfare and to reduce medication, in particular antibiotic, in animal husbandry. A vital communication between kingdoms in the mammalian gastro intestinal tract (GIT) is supposed by an exchange of OMVs/EVs that may interact in a triangular way between microbiome and host, majorly triggered by nutrition or medication. Hence the GIT transfer of species-specific small-RNAs via OMVs/EVs was investigated by a robust bioinformatical analysis on the containing small-RNAs. The spike in of non-OMV/EV controls to monitor the vesicular extraction and purification procedures and efficiency might be useful in functional studies. Nevertheless, those controls are novel, not well established and they will certainly vary depending on the spike-in model organism. Allan Stensballe (Aalborg University, Denmark) presented current challenges and pit-falls in proteogenomics and proteomic identification and characterisation of bacterial proteins from non-genome-sequenced bacteria.

A robust bioinformatic analysis was considered essential for this task. The addition of non-EV controls to monitor the EV extraction and purification procedures might be useful in functional studies. Nevertheless, those controls are still not well established and they will certainly vary depending on the bacterial model. Another important discussed issue to ensure reproducibility and result comparisons is the bacterial culture conditions that should be standardised, or at least clearly detailed in the reports. These conditions regard medium type (defined or complex), growth phase, cell viability, and others that might change EV production and features. Bacterial OMVs/EVs have already been tested as vaccines in numerous works, and therefore the purity and reproducibility of the material must be guaranteed for use in different biotechnology processes.

The OMVs are currently investigated as both vaccine adjuvants and therapeutics delivery vehicles. Whereas most published studies on EVs have focused on mammalian systems, the bacterial world could also have much to contribute to basic science and clinical applications. The Culture media used in most bacterial studies are so-called “rich” media, with complex components derived from biological sources such as yeast extract or meat extracts. A participant correctly noted that many bacterial strains could currently be grown in only one established culture medium. Since EVs or EV-like particles can be plentiful in rich media, defined “minimal” media culture conditions should be established unless (1) there is a reliable means to distinguish media EVs from those produced by the organism of interest or (2) the cultures produce so many EVs that culture media contaminants are only a small percentage of total EVs. However, for rich and minimal media alike, one must remember to assess the similarities and differences of the growth conditions with what the organism might encounter in its native environment. For the most widely used separation method, ultracentrifugation, participants noted that differences between a 100,000 x*g* ultracentrifuge pellet and other fractions have not been established for EVs from some organisms. Additional comparative studies of UC and other methods would be useful. For gradient ultracentrifugation, some preferred sucrose or iodixanol, and there was a difference of opinion about whether EVs could be stored in these gradient materials and how. One group stored iodixanol fractions at 4°C, while another froze them at −80°C. There is clearly room for methodology comparisons. Collection timing: phase, concentration, and cell death. While more EVs (per bacterium) might be present in stationary phase, the molecular profiles of vesicles released during different growth phases may be different (along with those of the releasing cells). This is consistent with the role of EVs in quorum sensing, as populations avoid overgrowth that may be harmful to themselves or a host organism. Therefore, the timing of harvest and population density (e.g. optical density) should be monitored, specified, and standardised for within- and between-study comparisons. Likewise, the ratio of dead and live cells should be reported, and populations with death over a pre-specified threshold (usually 5% or below) should not be used for EV separation.

The Participants identified an important question to solve: how to measure and report purity of EVs? An ideal purity measurement might include an EV-specific marker

or markers and a measure of total material or contaminating materials. In the mammalian EV literature, several metrics have been proposed. For example, protein concentration as a function of particle count [38] or EV protein marker(s) versus total protein [39]. Molecular "fingerprinting" assays are also in use [39]. For bacterial studies, generating a pan-bacterial EV antibody may very well be out of reach. However, existing anti-bacterial antibodies can be tested, and a Workshop participant reminded the group that generating a new antibody is a relatively simple process. If the perfect antibody to a single protein or strain cannot easily be identified, more general assays may also be helpful. LPS assays (gram-negative) and LTA assays (gram-positive) might identify properly separated EVs. Lipid/sterol measurements should be considered. Functional readouts. Whereas large quantities of material are often needed for omics profiling, minute amounts of EVs may elicit responses in functional assays. One proposal was to use macrophage responses to gauge bacterial EV potency. Care must of course be taken to ensure that signals are elicited by EVs and not by contaminating soluble factors. Contamination versus co-action, the issue of contamination was also found to be interesting from a conceptual perspective. Possibly, EVs do not act alone, but in concert with materials we might identify as contaminants. Is a surface-associated (but not integral) protein a contaminant? Must RNAs be located in the EV lumen to be EV RNAs? Is it possible to purify a population of EVs away from contaminants to the point that potency is lost? In that case, an EV prep might become "too pure"?

Fungi

EVs isolated from the culture supernatant have been characterised in human pathogenic species like *Cryptococcus neoformans, Paracoccidioides brasiliensis, Candida albicans, Malassezia sympodialis* and others, in addition to *Saccharomyces cerevisiae*. The heterogeneity in the population sizes and electron density suggests that different routes are involved in fungal EV biogenesis.

Before reaching the extracellular space, fungal EVs must traverse a thick cell wall that is mainly constituted of structural polysaccharides (glucans, chitin, and mannoproteins). *Cryptococcus* spp. also has a more external large capsule composed mainly of glucuroxylomannan (GXM) of high molecular mass and EVs not only traverse the capsule, but also contribute to its formation. Fungal EVs carry virulence factors within their cargo, which is constituted of a large variety of proteins, carbohydrate (glycogen, glucan, mannose oligosaccharides, α-galactosyl epitopes), small and messenger RNA, lipids, and melanin [35,40,41]. Virulence factors carried in fungal EVs include GXM, heat-shock proteins, superoxide dismutase, catalases, proteases, laccase, and urease [42,43].

At least 60% of the proteins seen in fungal EVs have also been described in the fungal cell wall, suggesting that they are due to the release of EV contents or to the transient presence of the vesicles in this compartment. Fungal EV biogenesis seems to involve the formation of ectosome, exosomes, and inverted macropinocytosis, as observed in electron microscopy images of wild type and mutant fungal cells. On the other hand, among the mechanisms proposed to explain how EVs cross the fungal cell wall, the most plausible envisions the formation of cell wall pores by the enzymatic action of cell-wall modifying enzymes contained in EVs [42,44,45]. The finding of cell wall remodelling and other active enzymes, like proteases, in fungal EVs corroborates this hypothesis.

In the ISEV meeting we had the chance to hear from Juliana Aparecida Rizzo Balancin (UFRJ, Brazil) about the characterisation of EVs in *Aspergillus fumigatus*, which is an opportunistic pathogen that lives in the mycelial form. EVs have been isolated from protoplasts and carried cytokine-inducing molecules, besides enzymes and glycans potentially involved in cell wall formation. We also had a talk from Karen Spadari Ferreira (UNIFESP, Brazil) on the participation of *Sporothrix brasiliensis* EVs in the murine innate response through the *in vitro* interaction with macrophages and dendritic cells and about the characterisation of the overall EV RNA content in various fungal species using RNA-seq. Rosana Puccia (UNIFESP, Brazil) showed the lipid and glycan composition and the presence of sRNA (small nucleolar RNA, transfer RNA and microRNA) in vesicles released by *P. brasiliensis* and *Paracoccidioides lutzii*. Allan Stensballe (Denmark) presented the isolation and characterisation of beer-derived EVs OMVs.

The discussions on issues related to fungal EV isolation and characterisation revealed that the fungal as the bacterial systems deserve attention on the standardisation of growth conditions, including culture media and growth phase choice to make the results comparable and trustable. Defined medium should be preferred over complex media and protein aggregates should be avoided. Current fungal EV isolation protocols are based on serial centrifugation and filtration, while density gradient ultracentrifugation and gel filtration have been proposed to be used routinely after that. The lack of general biomarkers has hampered the development of affinity-chromatography protocols and so the community should pay more attention to find markers for fungal EVs [11,40,42–44].

Protozoa

Parasites are known to shed and release EVs that function as cell-to-cell effectors in host–parasite interaction including regulation of the host immune system as well as in parasite–parasite communication. These observations have been corroborated for different Kinetoplastidae protozoan such as *Trypanosoma cruzi, Trypanosoma brucei* and *Leishmania* spp), Apicomplexa (*Toxoplasma gondii, Plasmodium falciparum), Amoebae, Giardia* and *Trichomonas vaginalis*. These parasite EVs contain a wide variety of molecules, including proteins, lipids, and RNAs (non-transcribed RNAs and microRNAs). They have been explored as biomarkers for diagnosis and disease monitoring, and as immune modulators to suppress or stimulate the immune system, vectors for drug delivery, vaccination, and therapeutic agents [1,46–54].

The talk of Adriana Oliveira Costa (UFMG, Brazil) highlighted the morphological characterisation and protein composition of EVs obtained by the first time from four different strains of *Acanthamoeba* ssp. The release of EVs of *Giardia intestinalis* was characterised by proteomic analysis by Ingrid Evans-Ossens (Instituto de Pesquisa Pelle Pequeno Príncipe, Brazil). Armando De Menezes-Neto (FIOCRUZ, Brazil) demonstrated EV enrichment from *Leishmania infantum* and *L. braziliensis* and the presence of major surface glycoconjugates such as GP63, a virulence factor of several *Leishmania* species [54]. Seminal findings reported by Olivier's group showed that *L. major* and *L. infantum* promastigotes release EVs *in vivo* in the midgut of the sand fly vectors and to play an important role in the transmission and propagation within the host [55]. Co-inoculation of *Leishmania* EVs and promastigotes was found to favour a Th17-dependent hyperinflammation paralleled with an exacerbated infection.

The compositional variability and the role of different *T. cruzi* isolates was illustrated by the groups of Ana Claudia Torrecilhas (UNIFESP, Brazil) and Rodrigo Pedro Soares (FIOCRUZ, Brazil), indicating a possible correlation with the different Chagas' disease profiles.

For Apicomplexa parasites, Vera Pereira-Chioccola (Adolfo Lutz Institute, Brazil) highlighted the presence of small RNA and microRNA in *T. gondii* tachyzoite EVs, which could modulate the host immune response. Portillo brought to light new data demonstrating that reticulocyte-derived EVs from natural malarial infection can signal mechanisms between the bone marrow and the spleen, thus revealing molecular mechanisms related to the clinical syndromes. In line with this, Patrícia Xander (UNIFESP, Brazil) showed that B-1 cells infected with *L. amazonensis* release vesicles that modulate macrophage functions.

The most discussed topic was regarding the different methodologies used to obtain and characterise EVs released from cultured parasites and from infected cells. This was particularly relevant since most EVs contained cell surface components and it was questioned which mechanisms are involved in EV release. It was proposed that identification of specific EV markers is still necessary in this field.

Helminths

Parasitic worms (helminths) are a constraint in livestock production and have major impact on the health of the one billion people infected. Infections are associated with profound suppression of host inflammatory responses and directing the immune responses towards regulation favouring parasite survival and chronic infections. Due to the complex nature of helminths and the multiple way they interact with the host immune system development of effective vaccines have failed to a large extend so far. As EVs released from helminths may provide fundamental new insight into the immunomodulatory properties of these worms and provide a novel entry point for vaccine development research groups have characterised EVs released from these organisms and started to explore their functions in host-parasite interactions. These studies have high level of complexity due to the presence of several compartments and the difficulty in differentiating between EVs from the parasite and the host [1,56,57]. To visualise the EV uptake by host cells Antonio Marcilla (Universitat de Valencia, Spain) stain the EVs and their uptake in intestinal IEC-18 cells can then be followed using confocal microscopy. Studies were presented on the characterisation of EVs released by adults of different trematode species, where several worm specific proteins as well as nucleic acids have been identified. Current data on EV composition, as well as preliminary data on their use in controlling the disease (i.e. in diagnosis, vaccination, and treatment) were shown, as well as preliminary studies on their possible therapeutic use in a murine model of ulcerative colitis.

Peter Nejsum (now at Aarhus University, Denmark) focused on EVs of nematodes of veterinary importance, the whipworm *Trichuris suis*, the giant round worm, *Ascaris suum*, and the nodular worm, *Oesophagostomum dentatum*. Due to related host physiology and closely related parasites species in humans, the pig-parasite model also serves as a good model for similar worm infection in humans. Eline Palm Hansen (Nejsum's group) presented evidence that EVs released from these species, contained miRNAs and were taken up by cells in culture.

In addition, to explore the ability of EVs to suppress proinflamatory responses in dendritic cells *in vitro* studies have been conducted.

Maria Eugenia Ancarola (University of Buenos Aires, Argentina) presented results on cestodes EVs, focusing on the characterisation of the vesicles produced by larval stages of *Taenia crassiceps* and *Mesocestoides corti*. The presence of particular miRNAs as well as specific proteins was presented, representing putative new targets for non-invasive diagnosis.

Concluding remarks: EVs in diagnostic, adjuvants, vaccine and biotechnological applications

During the presentations and discussions the workshop provided new perspectives on the role of EVs in host–pathogens interactions. This may pave the way for the development of new diagnostic tools, preventive strategies and to design new therapeutic approaches. A major outcome was the necessity to establish robust protocols for the isolation and characterisation of EVs from the very diverse group of pathogens, and importantly, the identification of specific EV markers for each species. Finally, the workshop created an environment for future collaborative studies between scientists working with different pathogens.

Acknowledgements

The authors are much indebted to the International Society for Extracellular Vesicles (ISEV) for help and support to the ISEV Brazil Workshop. We thank all those who presented results and participated in discussions, not all of whom are included as co-authors.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The event was also partially supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2016/12111-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). A.C.T is currently supported by FAPESP grant 2016/01917-3. R.P.S. is currently supported by FAPEMIG grant PPM-X 00102-16. P. X. is currently supported by FAPESP grant 2016/17245-4. A. M. is currently supported by Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana (Valencia, Spain) grant PROMETEO/2016/156.

ORCID

Antonio Marcilla http://orcid.org/0000-0003-0004-0531
Armando Menezes-Neto http://orcid.org/0000-0002-2003-908X
Kenneth Witwer http://orcid.org/0000-0003-1664-4233
Munira Muhammad Abdel Baqui http://orcid.org/0000-0002-7945-2899
Sergio Schenkman http://orcid.org/0000-0001-9353-8480
Rosana Puccia http://orcid.org/0000-0002-3332-0487
Peter Nejsum http://orcid.org/0000-0002-6673-8505
Paolo Bergese http://orcid.org/0000-0002-4652-2168

References

[1] Marcilla A, Martin-Jaular L, Trelis M, et al. Extracellular vesicles in parasitic diseases. J Extracell Vesicles. 2014;3:25040.
[2] Campos JH, Soares RP, Ribeiro K, et al. Extracellular vesicles: role in inflammatory responses and potential uses in vaccination in cancer and infectious diseases. J Immunol Res. 2015;2015:1–14.
[3] Tkach M, Théry C. Communication by extracellular vesicles: where we are and where we need to go. Cell. 2016;164:1226–1232.
[4] Cox FE. History of human parasitology. Clin Microbiol Rev. 2002;15:595–612.
[5] Schorey JS, Harding CV. Extracellular vesicles and infectious diseases: new complexity to an old story. J Clin Invest. 2016;126:1181–1189.
[6] Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200:373–383.
[7] Yanez-Mo M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015;4:27066.
[8] Smith VL, Jackson L, Schorey JS. Ubiquitination as a mechanism to transport soluble mycobacterial and eukaryotic proteins to exosomes. J Immunol. 2015;195:2722–2730.
[9] Barteneva NS, Maltsev N, Vorobjev IA. Microvesicles and intercellular communication in the context of parasitism. Front Cell Infect Microbiol. 2013;3:49.
[10] Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic (Copenhagen, Denmark). 2008;9:871–881.
[11] Da Silva TA, Roque-Barreira MC, Casadevall A, et al. Extracellular vesicles from Paracoccidioides brasiliensis induced M1 polarization in vitro. Sci Rep. 2016;6:35867.
[12] Schorey JS, Cheng Y, Singh PP, et al. Exosomes and other extracellular vesicles in host-pathogen interactions. EMBO Rep. 2015;16:24–43.
[13] Chaput N, Théry C. Exosomes: immune properties and potential clinical implementations. Semin Immunopathol. 2011;33:419–440.
[14] Martín-Jaular L, de Menezes-Neto A, Monguió-Tortajada M, et al. Corrigendum: spleen-dependent immune protection elicited by CpG adjuvanted reticulocyte-derived exosomes from malaria infection is associated with changes in T cell subsets' distribution. Front Cell Dev Biol. 2016;4:153.

[15] Meningher T, Lerman G, Regev-Rudzki N, et al. Schistosomal MicroRNAs Isolated From Extracellular Vesicles in Sera of Infected Patients: A New Tool for Diagnosis and Follow-up of Human Schistosomiasis. J Infect Dis. 2017;215:378–386.
[16] Paolini L, Zendrini A, Di Noto G, Busatto S, et al. Residual matrix from different separation techniques impacts exosome biological activity. Scientific Rep. 2016;6:23550.
[17] Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci U S A. 2016;113:E968–E977.
[18] Regente M, Corti-Monzón G, Maldonado AM, et al. Vesicular fractions of sunflower apoplastic fluids are associated with potential exosome marker proteins. FEBS Lett. 2009;583:3363–3366.
[19] Regente M, Pinedo M, Elizalde M, et al. Apoplastic exosome-like vesicles: a new way of protein secretion in plants? Plant Signal Behav. 2012;7:544–546.
[20] Raab-Traub N, Dittmer DP. Viral effects on the content and function of extracellular vesicles. Nat Rev Microbiol. 2017;15:559–572.
[21] Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol. 2014;29:116–125.
[22] Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. Proc Natl Acad Sci U S A. 2003;100:10592–10597.
[23] Nolte-'T Hoen E, Cremer T, Gallo RC, et al. Extracellular vesicles and viruses: are they close relatives? Proc Natl Acad Sci U S A. 2016;113:9155–9161.
[24] Ott DE. Purification of HIV-1 virions by subtilisin digestion or CD45 immunoaffinity depletion for biochemical studies. Methods Mol Biol. 2009;485:15–25.
[25] Dettenhofer M, Yu XF. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. J Virol. 1999;73:1460–1467.
[26] Sódar BW, Kittel Á, Pálóczi K, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Sci Rep. 2016;6:24316.
[27] van Dongen HM, Masoumi N, Witwer KW, et al. Extracellular vesicles exploit viral entry routes for cargo delivery. Microbiol Mol Biol Rev. 2016;80:369–386.
[28] Liao Z, Muth DC, Eitan E, et al. Serum extracellular vesicle depletion processes affect release and infectivity of HIV-1 in culture. Sci Rep. 2017;7:2558.
[29] Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol. 2015;13:605–619.
[30] van der Pol L, Stork M, van der Ley P. Outer membrane vesicles as platform vaccine technology. Biotechnol J. 2015;10:1689–1706.
[31] Sharpe SW, Kuehn MJ, Mason KM. Elicitation of epithelial cell-derived immune effectors by outer membrane vesicles of nontypeable Haemophilus influenzae. Infect Immun. 2011;79:4361–4369.
[32] Ren D, Nelson KL, Uchakin PN, et al. Characterization of extended co-culture of non-typeable Haemophilus influenzae with primary human respiratory tissues. Exp Biol Med (Maywood). 2012;237:540–547.
[33] Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol. 1999;181:4725–4733.
[34] Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev. 2005;19:2645–2655.
[35] Brown L, Wolf JM, Prados-Rosales R, et al. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol. 2015;13:620–630.
[36] Lee J, Kim OY, Gho YS. Proteomic profiling of Gram-negative bacterial outer membrane vesicles: current perspectives. Proteomics Clin Appl. 2016;10:897–909.
[37] Chutkan H, Macdonald I, Manning A, et al. Quantitative and qualitative preparations of bacterial outer membrane vesicles. Methods Mol Biol. 2013;966:259–272.
[38] Webber J, Clayton A. How pure are your vesicles? J Extracell Vesicles. 2013;2:19861.
[39] Reiner AT, Witwer KW, van Balkom BWM, et al. Concise review: developing best-practice models for the therapeutic use of extracellular vesicles. Stem Cells Transl Med. 2017;6:1730–1739.
[40] Nimrichter L, de Souza MM, Del Poeta M, et al. Extracellular vesicle-associated transitory cell wall components and their impact on the interaction of fungi with host cells. Front Microbiol. 2016;7:1034.
[41] Vargas G, Rocha JD, Oliveira DL, et al. Compositional and immunobiological analyses of extracellular vesicles released by Candida albicans. Cell Microbiol. 2015;17:389–407.
[42] Vallejo MC, Nakayasu ES, Matsuo AL, et al. Vesicle and vesicle-free extracellular proteome of Paracoccidioides brasiliensis: comparative analysis with other pathogenic fungi. J Proteome Res. 2012;11:1676–1685.
[43] Rodrigues ML, Nakayasu ES, Oliveira DL, et al. Extracellular vesicles produced by Cryptococcus neoformans contain protein components associated with virulence. Eukaryot Cell. 2008;7:58–67.
[44] Rodrigues ML, Nimrichter L, Oliveira DL, et al. Vesicular trans-cell wall transport in fungi: a mechanism for the delivery of virulence-associated macromolecules? Lipid Insights. 2008;2:27–40.
[45] Albuquerque PC, Nakayasu ES, Rodrigues ML, et al. Vesicular transport in Histoplasma capsulatum: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol. 2008;10:1695–1710.
[46] Trocoli Torrecilhas AC, Tonelli RR, Pavanelli WR, et al. Trypanosoma cruzi: parasite shed vesicles increase heart parasitism and generate an intense inflammatory response. Microbes Infect. 2009;11:29–39.
[47] Torrecilhas AC, Schumacher RI, Alves MJ, et al. Vesicles as carriers of virulence factors in parasitic protozoan diseases. Microbes Infect. 2012;14:1465–1474.
[48] Nogueira PM, Ribeiro K, Silveira AC, et al. Vesicles from different Trypanosoma cruzi strains trigger differential innate and chronic immune responses. J Extracell Vesicles. 2015;4:28734.
[49] Aline F, Bout D, Amigorena S, et al. Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. Infect Immun. 2004;72:4127–4137.

[50] Bhatnagar S, Schorey JS. Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. J Biol Chem. 2007;282:25779–25789.
[51] Pope SM, Lässer C. Toxoplasma gondii infection of fibroblasts causes the production of exosome-like vesicles containing a unique array of mRNA and miRNA transcripts compared to serum starvation. J Extracell Vesicles. 2013;2:22484.
[52] Regev-Rudzki N, Wilson DW, Carvalho TG, et al. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell. 2013;153:1120–1133.
[53] Coakley G, Maizels RM, Buck AH. Exosomes and other extracellular vesicles: the new communicators in parasite infections. Trends Parasitol. 2015;31:477–489.
[54] Atayde VD, Hassani K, da Silva Lira Filho A, et al. Leishmania exosomes and other virulence factors: impact on innate immune response and macrophage functions. Cell Immunol. 2016;309:7–18.
[55] Atayde VD, Aslan H, Townsend S, et al. Exosome secretion by the parasitic protozoan leishmania within the sand fly midgut. Cell Rep. 2015;13:957–967.
[56] Hansen EP, Kringel H, Williams AR, et al. Secretion of RNA-containing extracellular vesicles by the porcine Whipworm, Trichuris suis. J Parasitol. 2015;101:336–340.
[57] Coakley G, McCaskill JL, Borger JG, et al. Extracellular vesicles from a helminth parasite suppress macrophage activation and constitute an effective vaccine for protective immunity. Cell Rep. 2017;19:1545–1557.